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Identification of Sorbitol Transporters Expressed in the Phloem of Apple Source Leaves

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Sorbitol is a major photosynthetic product and a major phloem-translocated component in Rosaceae (e.g. apple, pear, peach, and cherry). We isolated the three cDNAs, *MdSOT3*, *MdSOT4*, and *MdSOT5* from apple (*Malus domestica*) source leaves, which are homologous to plant polyol transporters. Yeasts transformed with the *MdSOTs* took up sorbitol significantly. MdSOT3- and MdSOT5-dependent sorbitol uptake was strongly inhibited by xylitol and *myo*-inositol, but not or only weakly by mannitol and dulcitol. Apparent K_m values of MdSOT3 and MdSOT5 for sorbitol were estimated to be 0.71 mM and 3.2 mM, respectively. The protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), strongly inhibited the sorbitol transport. *MdSOT3* was expressed specifically in source leaves, whereas *MdSOT4* and *MdSOT5* were expressed in source leaves and also in some sink organs. *MdSOT4* and *MdSOT5* expressions were highest in flowers. Fruits showed no or only weak *MdSOT* expression. Although *MdSOT4* and *MdSOT5* were also expressed in immature leaves, *MdSOT* expressions increased with leaf maturation. In addition, in situ hybridization revealed that all *MdSOTs* were expressed to high levels in phloem of minor veins in source leaves. These results suggest that these MdSOTs are involved in sorbitol loading in Rosaceae.

Keywords: Apple (*Malus domestica*) — Phloem — Polyol — Rosaceae — Sorbitol transporter — Sugar alcohol.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DIG, digoxigenin; MFS, major facilitator superfamily; PCMBS, *p*-chloromercuribenzensulfonic acid; pI, isoelectric point; RFOs, raffinose family of oligosaccharides; TMD, transmembrane domain

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AB125646 (*MdSOT3*), AB125647 (*MdSOT4*), and AB125648 (*MdSOT5*).

Introduction

Sucrose is a major photosynthetic product and a major phloem-translocated component in most plants. However, some plants synthesize carbohydrates other than sucrose in source leaves and translocate them to sink organs. For instance, Cucurbitaceae plants synthesize raffinose family of oligosaccharides (RFOs), such as raffinose and stachyose, in source leaves and translocate them to sink tissues (Keller and Pharr 1996). Rosaceae, Apiaceae, and Plantaginaceae plants synthesize polyols (sugar alcohols) and use them as phloem-translocated compounds (Loescher and Everard 1996, Noiraud et al. 2001b). Polyols are low molecular weight, highly soluble, and non-reducing compounds; thus they are suitable for translocated compounds. Mannitol is a phloem-translocated component in celery (*Apium graveolens*) and sorbitol (D-glucitol) is that in *Plantago major* and in many Rosaceae trees.

Phloem loading is one of the most important features of source strength. Therefore, numerous studies have been conducted to understand this process (Turgeon 1996, Kühn et al. 1999, Lalonde et al. 2003). A sucrose transporter was first cloned from spinach (*Spinacia oleracea*) source leaves using the yeast expression system (Riesmeier et al. 1992) and this has triggered the isolation and characterization of this type of transporters from many plant species (Lalonde et al. 1999, Lemoine 2000, Kühn 2003). Riesmeier et al. (1993) showed that mRNA of the potato (*Solanum tuberosum*) sucrose transporter, *StSUT1*, was localized in the phloem of source leaves. They also showed that StSUT1 is essential for sucrose loading using antisense transgenic plants: the transgenic plants had less roots and tubers compared with wild-type plants and their source leaves accumulated high amounts of sugars and starch, since photoassimilate could not be exported from source leaves (Riesmeier et al. 1994). Similar results were obtained in experiments with the tobacco (*Nicotiana tabacum*) sucrose transporter, NtSUT1 (Bürkle et al. 1998). In *Arabidopsis*, the sucrose transporter, AtSUC2, was localized in companion cells (Truernit and Sauer 1995, Stadler and Sauer 1996) and the importance of AtSUC2 in phloem loading was supported by the characterization of its knock out-mutant (Gottwald et al. 2000). From these reports, sucrose phloem loading is thought to occur apoplastically mediated by a sucrose transporter.

Conversely, phloem loading of RFOs is thought to occur symplastically and its mechanism may be explained by the

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"polymer trap model" (Turgeon 1996). Sucrose synthesized in mesophyll cells of source leaves diffuses to specified companion cells, called intermediary cells, via plasmodesmata and sucrose is used as a substrate for RFOs synthesis. RFOs can move through plasmodesmata between intermediary cells and sieve elements, but not through the smaller plasmodesmata between intermediary cells and mesophyll or bundle sheath cells. As a result, RFOs accumulate in the intermediary cells and flow through the sieve tubes of the phloem.

Compared with sucrose and RFOs phloem loading, knowledge on polyol phloem loading is limited (Noiraud et al. 2001b). Noiraud et al. (2001a) isolated and characterized the mannitol transporter, AgMaT1, from celery. *AgMaT1* was expressed highly in source leaves and AgMaT1 was therefore suggested to be involved in phloem loading of mannitol. More recently, the two polyol transporter cDNAs, *PmPLT1* and *PmPLT2*, were isolated from *P. major*, whose major phloem-translocated component is sucrose but it also translocates sorbitol (Ramsperger-Gleixner et al. 2004). PmPLT1 and PmPLT2 proteins were localized specifically in companion cells of source leaf phloem, suggesting their importance in phloem loading of sorbitol.

Rosaceae includes many important fruit trees, e.g. apple, pear, peach, apricot, prune, and cherry and their major phloem component is sorbitol. In apple (*Malus domestica*) phloem, 70% of the translocated carbon was attributed to sorbitol (Klages et al. 2001). In peach (*Prunus persica*), sorbitol accounts for 60–90% of the carbon exported from source leaves and sorbitol concentration in the phloem sap was estimated to be 560 mM (Moing et al. 1997). In spite of the importance of sorbitol in Rosaceae, evidence of sorbitol transporters is limited to sink organs. Sorbitol transport activity was detected in apple fruit tissues (Berüter 1993, Berüter and Feusi 1995) and protoplasts isolated from apple fruit (Yamaki and Asakura 1988). Recently, cDNAs encoding sorbitol transporters (*PcSOT1* and *PcSOT2*) of sour cherry (*Prunus cerasus*) were identified (Gao et al. 2003). *PcSOT1* and *PcSOT2* were isolated from a fruit cDNA library and both were highly expressed in fruit.

With regard to sorbitol transporters in source leaves of Rosaceae, activity has only been detected in plasma membrane vesicles from peach leaves (Marquat et al. 1997) and the mechanism for phloem loading of sorbitol is still unclear (Noiraud et al. 2001b). In this article, we report on the cloning and characterization of sorbitol transporters from apple source leaves. They were expressed highly in the phloem of source leaves and may be involved in sorbitol phloem loading.

Results

Cloning of three sorbitol transporter cDNAs

To isolate cDNAs encoding sorbitol transporters, we performed RT-PCR and nested PCR using total RNA from apple source leaves and degenerate primers, designed based on conserved regions of the celery mannitol transporter (*AgMaT1*, AF215837, Noiraud et al. 2001a) and its homolog of sugar beet (*Beta vulgaris*, U64902). An amplified DNA fragment of ca. 580 bp was cloned and sequenced. Fourteen clones showed sequence homologies with *AgMaT1* and they were separated into three independent groups. Using mixed probes for the three groups, 22 positive clones were screened from 160,000 phage plaques of cDNA library constructed from apple source leaves. Two of these clones were independent and they were fully sequenced. Two cDNAs of putative sorbitol transporters from apple fruit, *SOT1* (AY237400) and *SOT2* (AY237401), were previously registered in the database, although their functions were not reported. Therefore, we designated the two cDNA clones from apple source leaves as *MdSOT3* and *MdSOT4*. Partial sequences of the other 20 clones were the same, thus the longest one was fully sequenced and designated as *MdSOT5*.

MdSOT3 was 1,866 bp long, potentially encoding a protein of 526 amino acid residues with a predicted molecular mass of 57 kDa and an isoelectric point (pI) of 9.22. *MdSOT4* was 2,155 bp long, potentially encoding a protein of 491 amino acid residues with a predicted molecular mass of 53 kDa and a pI of 6.71. *MdSOT5* was 1,961 bp long, potentially encoding a protein of 535 amino acid residues with a predicted molecular mass of 58 kDa and a pI of 8.97 (Fig. 1).

The consensus sequences for the sugar transporter subfamily, PESPRXL, (R/K)XGR(R/K), and PETQGRXXXE (Griffith et al. 1992) are present in MdSOTs (bold underlined in Fig. 1), although PET**Q**GRXXXE was changed to PET**H**GRXXXE in MdSOT5.

There were 12 transmembrane domains (TMDs) in MdSOT3 and MdSOT5 as predicted by MEMSAT (McGuffin et al. 2000). However, the predicted polypeptide of MdSOT4 was shorter in the N-terminal region and lacked the first transmembrane domain (TMD1) (Fig. 1). *MdSOT4* has 243 bases of upstream sequence from the putative initiation codon. Although this is far longer to encode the same length of N-terminal region as those of MdSOT3 and MdSOT5, there is no other initiation codon for right frame. Polyol transporters are members of the sugar transporter subfamily belonging to major facilitator superfamily (MFS). MFS is one of the largest transporter families in plants, animals, and microorganisms and it possesses a common structural motif of 12 TMDs (Marger and Saier 1993). The celery mannitol transporter, AgMaT1 (Noiraud et al. 2001a), was predicted to have 12 TMDs and MdSOT3 and MdSOT5 were also predicted to have 12 TMDs (Fig. 1). Conversely, sorbitol transporters from sour cherry, PcSOT1 and PcSOT2 were predicted to have only 11 TMDs by HMMTOP (Gao et al. 2003). This result might be an artifact of the hydropathy analysis and polyol transporters may well possess 12 TMDs as the same as sugar transporters.

Polyol transporters are members of the sugar transporter subfamily, but they are not closely related to known sucrose transporters, hexose transporters, and *myo*-inositol transporters

```
MdSOT3  MADRRAEENAVTGGPQNTSIEDFDPPMKPKTSKFAIACALLACTTSVLLGYDIGVMSGAS
MdSOT4  ----------------------------------------MTSILLGYDIGVMSGAA
MdSOT5  MADRTTDDNTVSSQPQKT-IADFDPPQKPKRNKYAFACAILASMTSILLGYDIGVMSGAA
                                                **:************:

MdSOT3  LYIQKNLKISDVQVEVLAGTLNIYSLLGSAFAGRTSDWIGRKYTIVLAGVIFLVGALLMG
MdSOT4  IYIEKDLKVTDTQIEILLGILNLYSLIGSAMAGRTSDWVGRRYTIVISGAIFFTGAILMG
MdSOT5  IYIKDDLKISDVEVEVLLGILNLYSLIGSAAAGRTSDWVGRRYTIVLAGAIFFVGALLMG
        :**:.:**::*.::*:* * **:***:*** *******:**:****::*.**:.**:***

MdSOT3  FATNYAFLMVGRFVAGVGVGYGMMIAPVYTAEISPASFRGFLTSFPEVFVNVGILLGYIA
MdSOT4  LSTNYTFLMCGRFVAGLGVGYALTIAPVYAAEVSPASSRGFLTSFPEVFVNVGILLGYIS
MdSOT5  FATNYSFLMFGRFVAGIGVGYALMIAPVYTAEVSPASSRGFLTSFPEVFINSGILLGYVS
        ::***:*** ******:****.: *****:**:**** **********:* ******::

MdSOT3  NYAFSKLPLHLGWRFMLGVGGVPAIFLTVGVLFMPESPRWLVMQGRLGDAKKVLQRTSES
MdSOT4  NYAFSFCALDVGWRLMLGVGAIPSVILAIGVLAMPESPRWLVMQGRLGDARQVLDKTSDS
MdSOT5  NYAFSKLPTHLGWRLMLGVGAIPSIFLAVGVLAMPESPRWLVMQGRLGDATRVLDKTSDS
        *****  . .:***:*****.:*:::*::*** ********************* :**::**:*

MdSOT3  KEECQLRLDDIKEAAGIPPHLNDDIVQVTKSSHGEGVWKELILHPTPAVRHILIAAVGIH
MdSOT4  KEESMLRLADIKEAAGIPEDCNDDIVQVTGHSHGEGVWKELFVHPTPTVLHIVIAALGFH
MdSOT5  KEESMLRLADIKEAAGIPEHCTDDVVQVPKRSQGQDVWKELLLHPTPAVRHILICAIGIH
        ***. *** ********* . .**:***.  *:*:.*****::****:* **:*.*:*:*

MdSOT3  FFEQASGIDTVVLYSPRIFAKAGITSSNHKLLATVAVGFTKTVFILVATFFLDKFGRRPL
MdSOT4  FFQQASGIDALVLYSPRVFEKAGITNSNQLLLCTVGVGLSKTVFTFVATFFLDRVGRRPL
MdSOT5  FFQQASGIDAVVLYSPRIFEKAGITNSDKKLLCTVAVGFVKTVFILVATFFVDKVGRRPL
        **:******::******:* *****.*:: **.**.**: **** :*****:*:.*****

MdSOT3  LLTSVGGMVFSLMFLGVGLTIVDHHK-GSVPWAIGLCMAMVYFNVAFFSIGLGPITWVYS
MdSOT4  VLTSMAGMVASLVCLGTSLTIVDQHEGARMTWAVVLCLCCVLAFVGFFSTGIGPIAWVYS
MdSOT5  LLASVAGMILSLTGLGLGLTIIDQNH-ERILWAAVLCLTMVLLYVAFFSIGMGPITWVYS
        :*:*:.**: **  ** .***:*::.   : **  **:  *   *.*** *:***:****

MdSOT3  SEIFPLKLRAQGVSIGVACNRVTSGVVSMTFISLYKAITIGGAFFLYAGISAAAWIFFYT
MdSOT4  SEIFPLRLRAQGCGMGVAVNRVMSGVLSMTFISLYKAITMGGAFFLYAAIGAVGWIFFFT
MdSOT5  SEIFPLKLRAQGCSLGVAMNRVVSGVLSMTFISLYEAITIGGAFFLYAAIASVAWVFFFT
        ******:***** .:*** *** ***:********:***:********.*.:.:*:**:*

MdSOT3  MLPETQGRTLEDTEVLFGKYHRWRKANAMLKERKQVDGDDNNNAQVF
MdSOT4  MLPETQGRTLEDMEVLFGKFHKWRKANKLLEKERQVARGDGGTSDPDNRVNLLA
MdSOT5  MLPETHGRTLEDMEVLFGKFHKWRKANALLKQKKNASHGDGITNNGQVQLGTRGQVN
        *****:****** ******:*:***** :*::.::.  .*. . :
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Fig. 1 Comparison of the deduced amino acid sequences of MdSOT3, MdSOT4, and MdSOT5. Identical amino acid residues, strongly similar ones, or weakly similar ones are indicated by an asterisk, colon, or period, respectively. The fine underlined sequences are putative membrane-spanning domains predicted by MEMSAT (McGuffin et al. 2000). The bold underlined sequences correspond to those conserved in the sugar transporter subfamily.

(Gao et al. 2003). Polyol transporter homologs were found not only in polyol loaders but also in non-polyol loaders, such as *Arabidopsis*, sugar beet, soybean (*Glycine max*), and rice (*Oryza sativa*). A phylogenetic tree constructed from a Clustal alignment of polyol transporter homologs showed that the MdSOTs were more closely related to the sorbitol transporters from sour cherry fruit and the putative ones from apple fruit rather than to the mannitol transporter from celery, AgMaT1, polyol transporters from *P. major*, PmPLTs, and other homologs (Fig. 2).

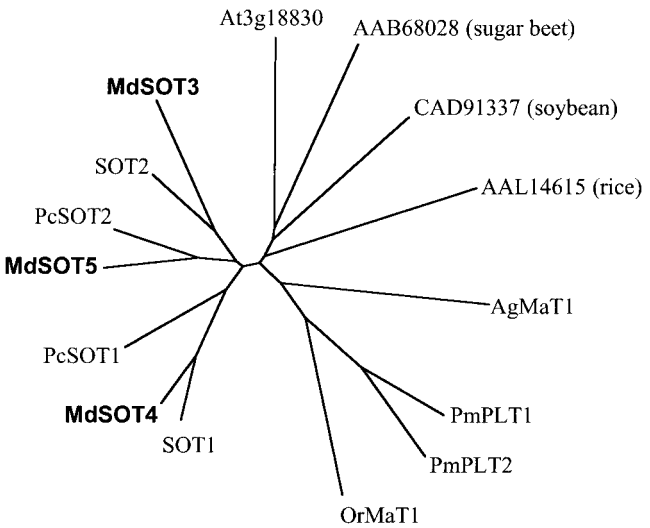

Fig. 2 Phylogenetic tree for polyol transporter homologs. Unrooted N-J Tree was constructed by CLUSTAL W. Protein IDs are as follows: sorbitol transporters from sour cherry, PcSOT1, AAO39267 and PcSOT2, AAM44082; putative sorbitol transporters from apple, SOT1, AAO88964 and SOT2, AAO88965; putative sugar transporter from *Arabidopsis*, At3g18830; probable sugar transporters from sugar beet, AAB68028; sorbitol-like transporter from soybean, CAD91337; putative sugar transporter from rice, AAL14615; mannitol transporter from celery, AgMaT1, AAG43998; polyol transporter from *Plantago major*, PmPLT1, CAD58709 and PmPLT2, CAD58710; putative mannitol transporter from *Orobanche ramosa*, OrMaT1, AAN07021.

Characterization of the MdSOT-dependent sorbitol transport in yeast

To characterize the sorbitol transport activity of MdSOT3, MdSOT4, and MdSOT5, their cDNAs were transformed in yeast (*Saccharomyces cerevisiae*) and sorbitol uptake into the yeast cells was determined. Yeast possesses a sorbitol dehydrogenase gene (*SDH1*). However, *SDH1* is only expressed when grown on sorbitol as the sole carbon source for at least 2 weeks (Sarthy et al. 1994). In our experiments, yeast was grown in a medium containing glucose or galactose, so that *SDH1* stayed repressed. Therefore, the yeast cells could not metabolize sorbitol and could be used for sorbitol uptake experiments. It was shown by thin-layer chromatography that [^{14}C]sorbitol taken up by yeast cells expressing PmPLT1 was not metabolized even after a 60-min incubation (Ramsperger-Gleixner et al. 2004).

Sorbitol uptake into yeast cells transformed with *MdSOT3*, *MdSOT4*, and *MdSOT5* was significantly increased compared with the control strain transformed with the empty pTV3e plasmid (Fig. 3). The MdSOT3- or MdSOT5-dependent sorbitol uptake was 62 or 17 times higher than the control, respectively. The transport activity of MdSOT4 was only 1.5 times higher than the control. This activity was too low to analyze; thus, only MdSOT3- and MdSOT5-dependent transport activities were further characterized.

The results of our competition and inhibition studies on the MdSOT3- and MdSOT5-dependent sorbitol uptake were

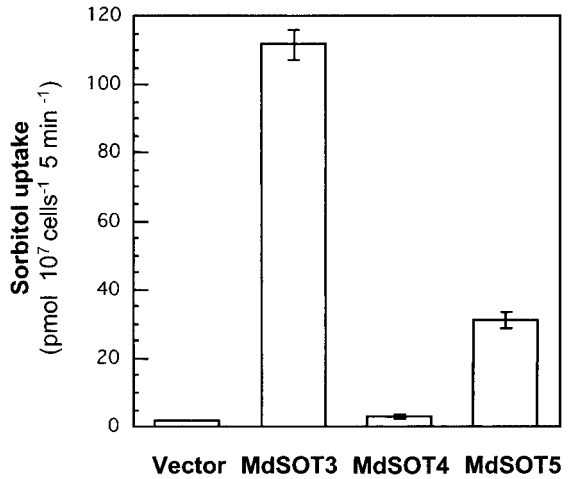

Fig. 3 Uptake of sorbitol by yeast cells expressing MdSOT3, MdSOT4, and MdSOT5. Uptake of 0.5 mM [^{14}C]sorbitol was measured at an external pH of 4.5. Yeast expressing MdSOT3, MdSOT4, or MdSOT5 was tested and compared with that harboring the empty vector. The results are means ± SE of three independent experiments.

quite similar (Table 1). Xylitol inhibited sorbitol uptake by 96 and 89%, respectively. *Myo*-inositol also inhibited sorbitol uptake by 67 and 56%, respectively. Other polyols, such as mannitol and dulcitol (D-galactitol) had less effect on sorbitol uptake. Glucose, fructose, and xylose inhibited sorbitol uptake by 66 to 36%. Sucrose had no effect on sorbitol uptake. The protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), strongly inhibited sorbitol uptake, suggesting that MdSOT3 and MdSOT5 are sorbitol/proton cotransporters. Recently, plant polyol transporters were shown to be proton-dependent by the measurement of an inward current in the presence of sorbitol and mannitol in *Xenopus* oocytes expressing PmPLT1 (Ramsperger-Gleixner et al. 2004). Sorbitol uptake was inhibited by the thiol reagent, $HgCl_2$. But, another thiol reagent, *p*-chloromercuribenzenesulfonic acid (PCMBS), had no significant effect on sorbitol uptake.

The concentration dependency of sorbitol transport by MdSOT3 and MdSOT5 showed Michaelis-Menten-type saturation kinetics (Fig. 4). From Lineweaver-Burk plots, apparent K_m values for sorbitol were estimated to be 0.71 mM and 3.2 mM for MdSOT3 and MdSOT5, respectively.

MdSOTs are expressed differentially in various organs and during leaf development

To investigate the gene expression patterns of *MdSOTs*, Northern blot analyses were performed using gene-specific RNA probes for the 3′ untranslated region of *MdSOTs*. Nucleotide identities among three probes were less than 28%. Expression of each *MdSOT* in various organs differed (Fig. 5). *MdSOT3* expression was detected only in source leaves, whereas *MdSOT4* and *MdSOT5* were expressed especially highly in flowers. *MdSOT4* was also expressed in mature fruit, stem, dormant buds, sink leaves, and source leaves. *MdSOT5* was expressed in dormant buds, sink leaves, and source leaves. No signal of *MdSOT* RNA was detected in seeds and young fruit.

Table 1 Effects of carbohydrates and inhibitors on sorbitol transport activities in yeasts expressing MdSOT3 and MdSOT5

Added compound	Sorbitol uptake (pmol 10^7 cells^{-1} 5 min^{-1})	
	MdSOT3 (%)	MdSOT5 (%)
None	169.0± 0.6 (100)	33.7±0.4 (100)
Sorbitol	18.3± 1.1 (11)	5.8±0.2 (17)
Mannitol	148.8± 2.1 (88)	30.1±0.5 (89)
Myo-inositol	55.9± 3.0 (33)	14.9±0.6 (44)
Dulcitol	165.8± 7.3 (98)	32.4±1.7 (96)
Xylitol	6.3± 0.1 (4)	3.9±0.2 (11)
Glucose	100.3± 1.7 (59)	18.7±0.7 (56)
Fructose	69.8± 2.0 (41)	13.9±1.1 (41)
Xylose	107.7± 3.3 (64)	11.3±0.5 (34)
Sucrose	169.4±68 (100)	35.8±1.9 (107)
CCCP	10.3± 0.7 (6)	4.3±0.2 (13)
$HgCl_2$	9.6± 0.1 (6)	4.2±0.2 (13)
PCMBS	147.3±28.6 (87)	37.0±5.3 (110)

Carrier [^{14}C]sorbitol concentration was 0.5 mM. Competing carbohydrates, CCCP, and $HgCl_2$ were added at concentrations of 5 mM, 50 μM, and 100 μM, respectively. Competing carbohydrates were added at the same time and inhibitors were added 30 s before addition of [^{14}C]sorbitol. The results are means ± SE of three independent experiments.

MdSOT expressions were also determined during leaf development (Fig. 6). Leaves were collected from a developing shoot and numbered consecutively: number 1 was the youngest and number 15 was the oldest leaf. Leaf numbers 1 to 4 were prior to full expansion. Expression patterns of *MdSOT4* and *MdSOT5* were similar, i.e., they were high in youngest and older leaves, but relatively low in the intermediate stages of leaf development. *MdSOT3* expression was undetectable in young leaves and increased with leaf development and was highest in mature leaves.

MdSOTs are expressed in the phloem of minor veins in source leaves

Because all three *MdSOTs* were expressed in source leaves, they might be involved in sorbitol phloem loading. To test this hypothesis, tissue localization of *MdSOT* mRNA was determined by in situ hybridization. When gene specific antisense RNA probes for *MdSOT3*, *MdSOT4*, and *MdSOT5* were hybridized to sections from apple source leaves, strong signals were detected in the phloem of minor veins (Fig. 7B–D). These signals are not unspecific, since no signal was detected in the control using a sense probe (Fig. 7A). No significant signal of *MdSOTs* was found in the xylem, bundle sheath, parenchyma, or epidermis.

Discussion

Apple sorbitol transporters and their homologs

MdSOT3, MdSOT4, and MdSOT5 from apple source leaves are more closely related to polyol transporter homologs from Rosaceae rather than to those from other families (Fig. 2). This suggests that the Rosaceae sorbitol transporter homologs diverted after the Rosaceae had evolved from other families. MdSOT3 and MdSOT4 from 'Fuji' apple leaves have close relationships with the putative sorbitol transporters from 'Mutsu' apple fruit, SOT2 and SOT1, respectively (Fig. 2). However, amino acid identity between MdSOT3 and SOT2, and that between MdSOT4 and SOT1 were only 79% and 89%, respectively. The difference might be higher than the difference between varieties and at least five sorbitol transporter paralogues might exist in apple, i.e., *SOT1*, *SOT2*, *MdSOT3*, *MdSOT4*, and *MdSOT5*.

Transport activities of MdSOTs and other polyol transporters

Sorbitol uptake into yeast transformed with *MdSOT3*, *MdSOT4*, and *MdSOT5* was significantly higher than that of the control (Fig. 3). However, MdSOT4-dependent uptake was very low and this may be caused by its protein structure, i.e., the lack of TMD1 (Fig. 1). The three-dimensional structure of the MFS showed that TMD1 forms a substrate-translocation pore together with TMD4, 7, and 10 (Hirai et al. 2002, Abramson et al. 2003, Huang et al. 2003). This illuminates the importance of TMD1 for substrate-translocation and the lack of TMD1 in MdSOT4 may be responsible for its low activity.

The mannitol transporter from celery (AgMaT1) and the polyol transporters from *P. major* (PmPLT1 and PmPLT2) were characterized as unspecific polyol transporters. AgMaT1-dependent mannitol transport was inhibited by dulcitol, sorbitol, *myo*-inositol, and xylitol (Noiraud et al. 2001a). PmPLT1- and PmPLT2-dependent sorbitol transport was inhibited only little by dulcitol and inositol, and mannitol inhibited only the PmPLT1-dependent transport significantly. However, both PmPLTs catalyzed not only [^{14}C]sorbitol but also [^{14}C]mannitol transport. The authors suggested that the weak inhibitory effects are caused by the higher K_m value of PmPLT2 and concluded that PmPLTs are polyol transporters of low specificity (Ramsperger-Gleixner et al. 2004).

Conversely, sorbitol transporters from sour cherry (PcSOT1 and PcSOT2) and apple (MdSOT3 and MdSOT5) showed relatively high specificities for sorbitol. However, the substrate specificities of MdSOT3 and MdSOT5 differed from those of PcSOT1 and PcSOT2. PcSOT1- and PcSOT2-dependent sorbitol transport was inhibited by mannitol, but only weakly by xylitol (Gao et al. 2003). MdSOT3- and MdSOT5-dependent sorbitol transport was inhibited by xylitol and *myo*-inositol, but not or only weakly by mannitol and dulcitol (Table 1).

Glucose, fructose, and xylose inhibited sorbitol uptake depending on MdSOT3 and MdSOT5 (Table 1). Similar inhibitory effects of glucose and fructose were observed for the polyol transport of PcSOT1, PcSOT2, and AgMaT1 (Noiraud et al. 2001a, Gao et al. 2003). The presence of MdSOTs did not significantly enhance [^{14}C]glucose uptake in LBY416 (data not shown), suggesting that MdSOTs cannot transport hexoses.

PCMBS, which is a thiol reagent known to act as an inhibitor for certain sugar transporters, had no significant effect on the MdSOT3- and MdSOT5-dependent sorbitol transport in yeast (Table 1). This is not uncommon for polyol transporters. PCMBS did not or only slightly inhibit AgMaT1, PcSOT1, PcSOT2, and PmPLT2 (Noiraud et al. 2001a, Gao et al. 2003, Ramsperger-Gleixner et al. 2004). One exception concerns the PmPLT1-dependent sorbitol transport, which was strongly inhibited by PCMBS (Ramsperger-Gleixner et al. 2004). By the comparison of the Cys residues in polyol transporters, these authors suggested that Cys_{61} in PmPLT1 was responsible for the inhibition by PCMBS. Cys_{61} is located in the predicted first extracellular loop. MdSOT3 and MdSOT5 lack such a Cys residue, which is in line with their hypothesis. We here propose an additional hypothesis. In this study, sorbitol uptake was strongly inhibited by another thiol reagent, $HgCl_2$ (Table 1). This suggests the existence of another Cys residue responsible for the inhibition by Hg^{2+} and this Cys may be present inside the cell. Because PCMBS is not membrane permeable, it can only bind to extracellular Cys, such as Cys_{61} in PmPLT1. Hg^{2+} is membrane permeable and it could also bind to intracellular Cys.

Various ranges of K_m values for sorbitol transport in tissues or isolated membranes have been reported. The K_m value for sorbitol uptake into apple fruit tissues was between 35 and 55 mM (Berüter 1993), that for apple fruit protoplasts was 3.6 mM (Yamaki and Asakura 1988), and that for plasma membrane vesicles from peach leaves was 0.67 mM (Marquat et al. 1997). Recent research using the yeast expression system showed the existence of both low affinity and high affinity polyol transporters (Noiraud et al. 2001a, Gao et al. 2003, Ramsperger-Gleixner et al. 2004): PcSOT1 (K_m = 0.6 mM for sorbitol), PcSOT2 (K_m = 0.8 mM for sorbitol), and AgMaT1 (K_m = 0.3 mM for mannitol) are considered high affinity transporters and PmPLT1 (K_m = 12 mM for sorbitol) and PmPLT2 (K_m for sorbitol is too high to calculate) are considered low affinity transporters. The K_m value of MdSOT3 for sorbitol (0.71 mM) is almost the same as the K_m values for plasma membrane vesicles from peach leaves, PcSOT1 and PcSOT2 from sour cherry, and MdSOT3, therefore, is clearly a high affinity transporter. The K_m value of MdSOT5 for sorbitol (3.2 mM) is similar to that of apple fruit protoplasts and it was considered between high affinity and low affinity. The different K_m values of MdSOT3 and MdSOT5 may reflect their different physiological roles.

Different gene expressions of MdSOTs suggest different functions

The different physiological roles of MdSOTs were also suggested by their gene expression patterns in various organs

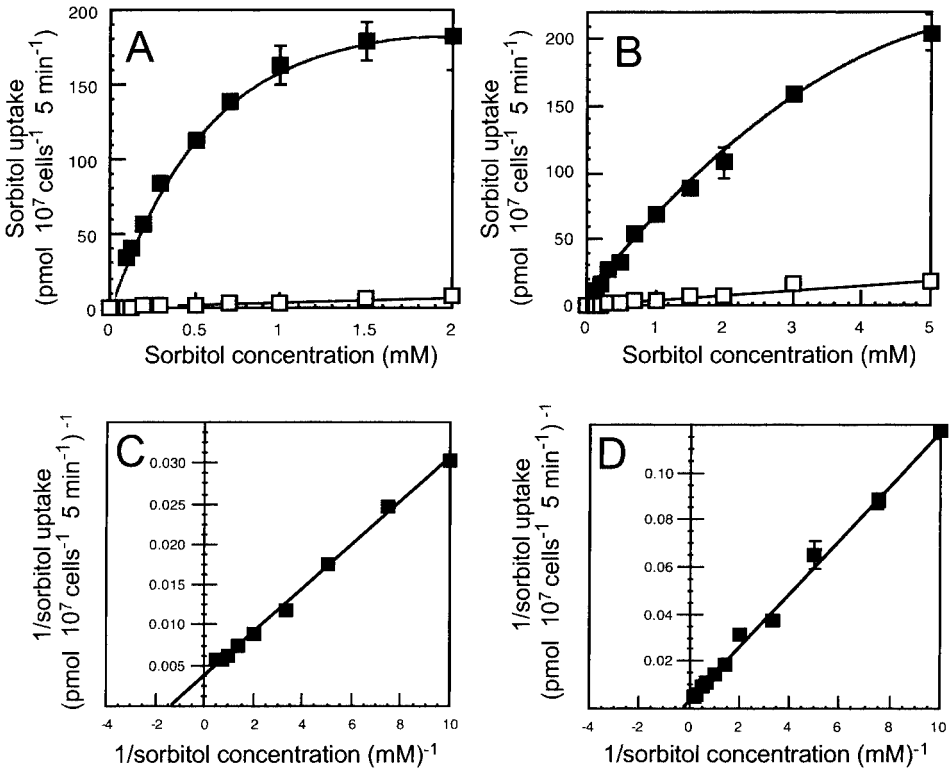

Fig. 4 Concentration dependence of sorbitol uptake in yeast cells expressing MdSOT3 (A) and MdSOT5 (B). Open squares in A and B represent sorbitol uptake by control yeast cells transformed with the empty vector. (C) and (D) show Lineweaver-Burk plot of the uptake depending on MdSOT3 or MdSOT5, respectively. Apparent K_m values for sorbitol of MdSOT3 and MdSOT5 were 0.71 mM and 3.2 mM, respectively. The results are means ± SE of three independent experiments.

and during leaf development (Fig. 5, 6). The expression of *MdSOT4* and *MdSOT5* was highest in flowers. Since flowers are one of the most active sink organs, MdSOT4 and MdSOT5 might be important for sorbitol unloading to act as a substrate for the active metabolism in flowers. High *MdSOT5* expression and low *MdSOT4* expression were detected in dormant buds. Polyols are protectants against environmental stresses, such as low temperature and drought (Loescher and Everard 1996). Apple trees store sorbitol in trunks, stems, and dormant buds for cold-hardiness in winter. MdSOT5 and MdSOT4 in dormant buds might be responsible for sorbitol accumulation during cold acclimation.

PcSOT1 and *PcSOT2* were highly expressed in sour cherry fruit (Gao et al. 2003). However, no or only low expression of *MdSOTs* was detected in apple fruit (Fig. 5). This indicates that MdSOTs may not play any role in apple fruit. Two cDNAs of putative sorbitol transporter (*SOT1* and *SOT2*) were isolated from apple fruit. Although their expression patterns and functions have not been reported, they might be responsible for fruit development and sugar accumulation in apple fruit.

The expression of *MdSOT4* and *MdSOT5* was high in youngest leaves, then it decreased and increased again with leaf maturation (Fig. 6). This indicates that MdSOT4 and MdSOT5 play different roles in sink and source leaves; they may function in sorbitol import in sink leaves and in sorbitol export in source leaves. Although *PcSOT1* from sour cherry fruit was also expressed in developing leaves, the expression decreased with leaf development and never increased. Therefore, the function of PcSOT1 differs from that of MdSOT4 and MdSOT5; PcSOT1 is important for leaf development, but it may not be important in sorbitol loading. Interestingly, *MdSOT3* expression was detected only in source leaves (Fig.

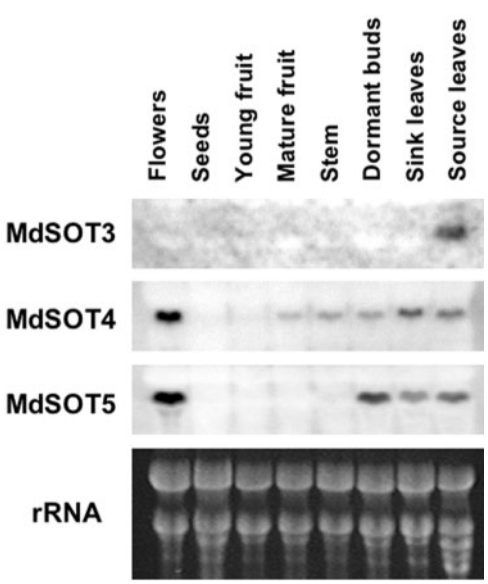

Fig. 5 Northern blot analysis for *MdSOT3 MdSOT4*, and *MdSOT5* in various apple organs. Ten μg of total RNA from flowers, seeds, young fruit, mature fruit, stem, dormant buds, sink leaves, and source leaves of apple were subjected to Northern blot analysis with gene specific RNA probe for each *MdSOT*.

5). It was undetectable in young leaves and increased with leaf development (Fig. 6). This expression pattern is similar to that of the NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH), which is a key enzyme of sorbitol synthesis, in peach leaf development (Sakanishi et al. 1998). Similar expression pattern of S6PDH was observed in pear leaf development (unpublished data). We do not have such data in apple, if it is similar to peach and pear, synchronized gene expressions between key enzyme of sorbitol synthesis and sorbitol transport are interesting and quite reasonable.

MdSOTs and sorbitol phloem loading in Rosaceae

MdSOTs were all expressed in source leaves (Fig. 5). In addition, in situ hybridization revealed that three *MdSOTs* were expressed to high levels specifically in the phloem of minor veins in source leaves (Fig. 7). These results show that MdSOT3, MdSOT4, and MdSOT5 may all play some roles in phloem loading of sorbitol in source leaves. It has been discussed that phloem-localized sucrose transporters may function as a mechanism for retrieval of sucrose (Lalonde et al.

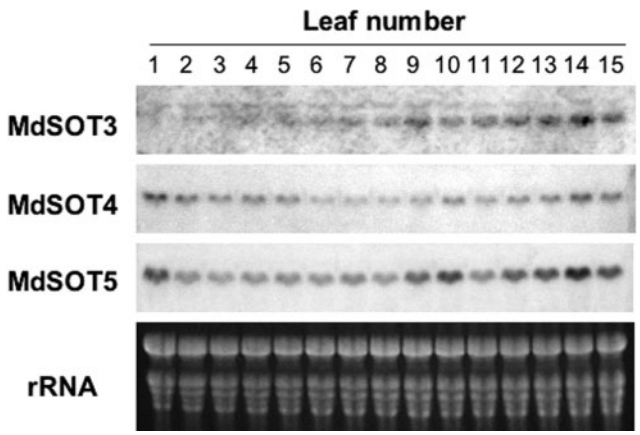

Fig. 6 Northern blot analysis for *MdSOT3*, *MdSOT4*, and *MdSOT5* during apple leaf development. Leaves were collected from developing shoot and numbered from shoot apex (leaf number 1 was the youngest and number 15 was the oldest). Ten μg of total RNA were subjected to Northern blot analysis with gene specific RNA probe for each *MdSOT*.

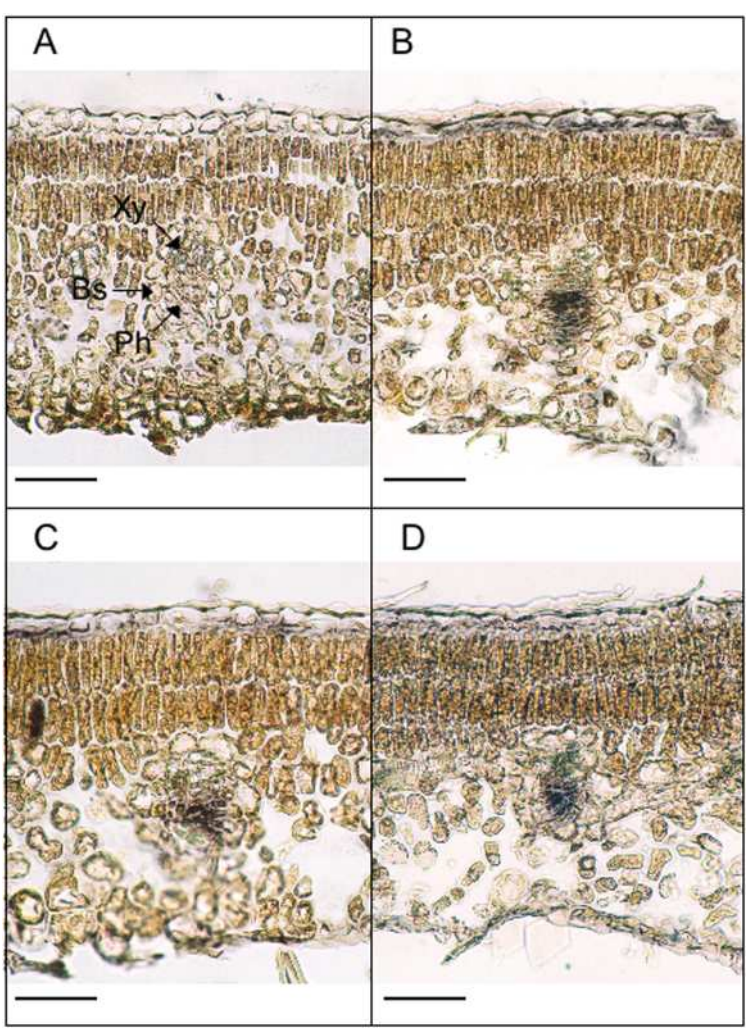

Fig. 7 In situ localization of *MdSOT* mRNAs in apple source leaves. Sections from apple source leaves were hybridized to DIG-labeled sense RNA of 3′ untranslated region of *MdSOT3* (A), antisense RNA of 3′ untranslated region of *MdSOT3* (B), that of *MdSOT4* (C), or that of *MdSOT5* (D). Xy, xylem; Ph, phloem; Bs, bundle sheath. Scale bars = 200 μm.

1999). This function is important in major veins of source leaves and vascular systems of petioles and stems rather than in minor veins of source leaves. *MdSOT* expressions were detected in the minor veins, which are of the 6th or 7th order. In major veins, although very weak *MdSOT* expressions were detected in xylem, no significant *MdSOT* expression was detected in phloem (data not shown). These results suggest that MdSOTs are involved in phloem loading rather than in retrieval of sorbitol. MdSOT4-dependent sorbitol transport in yeast was unclear (Fig. 3), but MdSOT3 and MdSOT5 transported sorbitol and their apparent K_m values were different (Fig. 4). Different affinity transporters in the phloem of source leaves could allow a regulation of phloem loading at different sorbitol concentrations.

Recently, Ramsperger-Gleixner et al. (2004) reported that two different polyol transporters (PmPLT1 and PmPLT2) and one sucrose transporter (PmSUC2) localized specifically to companion cells in *P. major* source leaves. *P. major* loads mainly sucrose and additionally sorbitol into the phloem. Plantaginaceae have closed-type phloem structures (less plasmodesmal connections between mesophyll and companion cells, Gamalei 1989). Therefore, sucrose and sorbitol loading is thought to occur apoplastically and the presence of sucrose and sorbitol transporters in companion cells is quite reasonable. Conversely, the major phloem component of most of the woody Rosaceae is sorbitol. The phloem structure of woody plants is generally of the intermediate-type (moderate numbers of plasmodesmal connections between mesophyll and companion cells) and the phloem structures of Rosaceae trees were also shown to be of the intermediate-type (Gamalei 1989, Turgeon et al. 2001). Therefore, the mechanism for phloem loading of sorbitol in Rosaceae trees may differ from Plantaginaceae and this has been a matter of debate, i.e. 'apoplastic' vs. 'symplastic' (Noiraud et al. 2001b). In peach trees, the sorbitol concentration of the phloem sap was very close to that of the other leaf tissues (Moing et al. 1997), suggesting sorbitol loading to occur symplastically through plasmodesmata. Conversely, the same article showed that the sorbitol concentration in phloem exudates from source leaves was reduced by PCMBS treatment, suggesting that sorbitol is loaded by a PCMBS-sensitive transporter. However, the activities of sorbitol transporters from Rosaceae in yeast were not or only slightly inhibited by PCMBS (Table 1, Gao et al. 2003). This complicated situation is reminiscent of previous debates concerning the mode of phloem loading of sucrose and the finding of the sucrose transporter genes expressed in the phloem triggered off the clarification of the mechanism of sucrose loading as described above.

In this study, we identified sorbitol transporter genes expressed in the phloem of minor vein in apple source leaves. This is the first report of sorbitol transporter genes expressed in the phloem of Rosaceae and suggests the involvement of sorbitol transporters in sorbitol phloem loading in Rosaceae. Further investigation of *MdSOTs* will clarify the mechanism of sorbitol phloem loading in Rosaceae, as was the case of sucrose phloem loading.

Materials and Methods

Plant materials

Samples were collected from apple (*Malus domestica* Borkh cv. Fuji) tree cultivated in the orchard of Nagoya University. For molecular cloning and construction of cDNA library, source leaves were collected on June 6, 2001. For Northern blot analysis, sink and source leaves were collected on April 25 and June 4, 2002, respectively. Young and mature fruits were harvested on June 8 and October 30, 2002, respectively. Flowers, shoots, or dormant buds, were collected on April 14, June 4, 2002, and January 22, 2003, respectively. Seeds were collected from mature fruit. Samples for leaf developmental experiment were collected from developing shoots on June 4, 2002. Fruit flesh and other samples were frozen in liquid nitrogen and stored at –80°C. For in situ hybridization analysis, source leaves were collected on September 30, 2003 and fixed immediately.

Construction of cDNA library and molecular cloning

Total RNA was isolated from source leaves as described previously (Suzuki et al. 1999). Poly $(A)^+$ RNA was isolated from total RNA using Takara Oligotex-dT30<super> (Takara Bio. Inc., Otsu, Japan). cDNA library was ligated into Uni-Zap XR phage vector (Stratagene, La Jolla, CA, U.S.A.), and then packed using Gigapack Gold packaging extract (Stratagene).

cDNA fragments of sorbitol transporter were amplified with total RNA from source leaves by RT-PCR and nested PCR. Degenerate primers were designed based on conserved region of celery mannitol transporter (*AgMaT1*, AF215837, Noiraud et al. 2001a) and its homologue of sugar beet (U64902). The primers for first PCR and those for nested PCR were (forward: SR5A, 5′-CCTAARAGRAAYAAGTWT-GCTTTTGCTTGTGCT-3′ and reverse: SR3A, 5′-CCCTCCRAACA-AAAAGAAKGCACCACC-3′) and (forward: SR5A and reverse: SR3B, 5′-AACRAGCCAMCGYGGKGACTCWGGCAT-3′), respectively. An amplified DNA fragment of ca. 580 bp was ligated into pT7Blue plasmid vector (Novagen, Darmstadt, Germany) according to the manufacturer's instructions and sequenced.

The cDNA fragments were labeled with digoxigenin (DIG) using DNA-DIG Labeling Kit (Roche, Mannheim, Germany). cDNA library was screened by the standard hybridization method. Positive clones were in vivo excised into the pBluescript SK(–) plasmid (Stratagene) and sequenced.

Northern blot analysis

Total RNA was isolated from various apple organs according to the method by Suzuki et al. (1999). Total RNA (10 μg) was separated on 1.0% (w/v) agarose gels containing formaldehyde and transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

To prepare gene specific RNA probes, 3′ untranslated region of *MdSOTs* were amplified by PCR with following primers. *MdSOT3*: forward: 5′-GCAAGTTGATGGTGACGACAACAA-3′ and reverse: 5′-CAGAGATGCATAATACGACTCACTATAGGGAGATCTGCAAG-GTATGTCCTATA-3′. *MdSOT4*: forward: 5′-CTCGTGGTGATGGTG-GCACCAGTG-3′ and reverse: 5′-CAGAGATGCATAATACGACTCA-CTATAGGGAGATCTAATACGAAATGTTTTAT-3′. *MdSOT5*: forward: 5′-CAAGCAGAAAAAGAATGCCAGTCA-3′ and reverse: 5′-CAGAGATGCATAATACGACTCACTATAGGGAGATGTAAAATAC-ACATGGAATT-3′. All these reverse primers contain T7 polymerase promoter sequence at 5′-end to synthesize antisense RNAs. Antisense

RNA probes were prepared by in vitro transcription using DIG-RNA Labeling Kit (Roche). Hybridization was performed at 68°C overnight. The membrane was washed twice in 0.1× SSC containing 0.1% (w/v) SDS at 68°C for 15 min and subjected to detection of DIG-labeled probe using anti-DIG antibody (Roche) and CDP-star (TROPIX, Bedford, MA, U.S.A.).

In situ hybridization

Leaf tissues were fixed with FAA medium (5% (v/v) acetic acid, 3.7% (v/v) formaldehyde, 50% (v/v) ethanol) for 16 h at 4°C after vacuum infiltration. The tissue was dehydrated through an alcohol series, embedded into paraffin, and sliced into 6-μm-thick sections. The sections were placed on aminopropyl-triethoxy silane (APS) coated micro slide glass (Matsunami, Kishiwada, Japan), deparaffinized in xylene, and hydrated by passing through an alcohol series. The sections were incubated in 0.2 M HCl for 20 min, in 100 mM triethanolamine containing 0.25% (v/v) acetic anhydride for 10 min, and in 2× SSC for 5 min. The sections were dehydrated through an alcohol series to 100% ethanol and vacuum dried. The same DIG-labeled RNA probes used in Northern blot analysis were used as antisense probes. For control, DIG-labeled sense RNA for 3′ untranslated region of *MdSOT3* was synthesized with following primers by the same method described above. Sense: forward: 5′-CAGAGATGCATAATACGACTCACTATAGGGAGAGCAAGTTGATGGTGACGACA-3′ and reverse: 5′-GTTTTCCCAGTCACGACG-3′. The sense primer contains T7 polymerase promoter sequence at 5′-end to synthesize sense RNA. U19 sequence of cloning vector was used for reverse primer. Hybridization was carried out at 55°C for 16 h in a humid box. After hybridization, the sections were washed with 4× SSC 4 times at 55°C for 10 min, treated with 25 μg ml^{-1} ribonuclease A at 37°C for 30 min, and then washed with 2× SSC twice at 55°C for 30 min, and with 0.1× SSC twice at 55°C for 30 min. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates for alkaline phosphatase-conjugated anti-DIG antibody.

Heterologous expression of MdSOTs in yeast

MdSOTs were constructed in a pTV3e plasmid and expressed in yeast (*Saccharomyces cerevisiae*) strain LBY416 (*MAT hxt2::LEU2 snf3::HIS3 gal2 lys2 ade2 trp1 his3 leu2 ura3*) according to the method by Toyofuku et al. (2000). LBY416 is the yeast strain, whose monosaccharide transport activity is kept low, since three monosaccharide transporter-related genes, *HXT2*, *GAL2*, and *SNF3* are disrupted (Kruckeberg and Bisson 1990). pTV3e is a multicopy plasmid and DNA subcloned in it was expressed under the control of the *GAL2* promoter (Nishizawa et al. 1995). Coding region of *MdSOTs* was amplified by PCR with *Pfu* DNA polymerase (Stratagene) and following primers. *MdSOT3*: forward: 5′-ATGGCTGAATTCAGGGCGGAAGAGAATG-3′ and reverse: 5′-TATCGATTAGAAAACTTGGGCATTGTTG-3′. *MdSOT4*: forward: 5′-ATGACTGAATTCCTGCTGGGTTATGA-3′ and reverse: 5′-CATCGATTAAGCCAAGAGGTTCACCCTG-3′. *MdSOT5*: forward: 5′-ATGGCTGAATTCACAACTGACGATAATAC-3′ and reverse: 5′-AATCGATTAATTAACTTGCCCTCTTGTTC-3′. PCR products containing *Eco*RI or *Cla*I site on both sites were ligated into pTV3e vector. After checking their sequences, *MdSOT*-pTV3e was introduced into LBY416.

Sorbitol uptake in the yeasts expressing MdSOTs

Uptake of sorbitol into yeast cells was examined according to the procedures described previously (Kasahara et al. 1997, Toyofuku et al. 2000). Yeast cells were grown in SD medium [2% (w/v) glucose, Burke et al. 2000] to early logarithmic phase, transferred to SGal medium [2% (w/v) galactose, Burke et al. 2000] and grown for 16 h to express MdSOTs. Cells were washed with 50 mM MES-NaOH (pH 6.0) containing 2 mM $MgSO_4$, and suspended to ca. 10^8 cells ml^{-1} in 50 mM MES-NaOH (pH 4.5) containing 2 mM $MgSO_4$. Aliquots (180 μl) of the cell suspension and 20 μl of sorbitol solution, containing 925 kBq ml^{-1} D-[U-^{14}C]sorbitol (Amersham Pharmacia Biotech), were mixed and incubated at 30°C. Reaction was stopped by the addition of 5 ml of cold stopping solution (50 mM MES-NaOH (pH 6.0) containing 2 mM $MgSO_4$ and 100 μM $HgCl_2$), filtrated on glassfiber filters (GF/F, Whatman, Kent, U.K.), and washed with 15 ml of cold stopping solution. The radioactivity incorporated in the cells was determined with a liquid scintillation counter (LSC-5100, ALOKA, Tokyo, Japan).

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